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Understanding, improving and using green fluorescent proteins

Andrew B. Cubitt, Roger Heim, Stephen R. Adams, Aileen E. Boyd, Larry A. Gross and Roger Y. Tsien

Green fluorescent proteins (GFPs) are presently attracting tremendous interest as the first general method to create strong visible fluorescence by purely molecular biological means. So far, they have been used as reporters of gene expression, tracers of cell lineage, and as fusion tags to monitor protein localization within living cells. However, the GFP originally cloned from the jellyfish *Aequorea victoria* has several nonoptimal properties including low brightness, a significant delay between protein synthesis and fluorescence development, and complex photoisomerization. Fortunately, the protein can be re-engineered by mutagenesis to ameliorate these deficiencies and shift the excitation and emission wavelengths, creating different colors and new applications.

THE TWO BEST-CHARACTERIZED green fluorescent proteins (GFPs) are from marine invertebrates: a Pacific Northwest jellyfish, *Aequorea victoria*, and a sea pansy from the Georgia coastline, *Renilla reniformis*[1]. Other members of this coelenterate sub-phylum Cnidaria contain fluorescent proteins which remain to be characterized[2,3]. *Aequorea* and *Renilla* GFPs each transmute blue chemiluminescence from a distinct primary photoprotein into green fluorescence. The first written report of such bioluminescence was from Pliny the Elder in the first century AD, who observed the bright glow of certain jellyfish present in the Bay of Naples[4]. His early development of glowing slime that could be scraped from these organisms and used to make various articles luminescent was abruptly terminated by the eruption of Vesuvius in AD79. More recently, biochemical characterization of the GFPs in the labs of Blinks, Cormier, Hastings, Johnson and Shimomura, Prendergast and Ward began in the 1960s and culminated in the cloning of a cDNA (*gfp10*) for *Aequorea* GFP by Prasher *et al.*[5] Chalfie *et al.*[6] then triggered an enormous upsurge of interest in GFP by showing that expression of the cloned gene produces fluorescent protein in a variety of cell types, as also reported by Inouye and Tsuji[7].

As yet there is no definitive explanation as to why these organisms evolved bioluminescence or fluorescent proteins[3]. GFPs probably serve not just to shift the output color from blue to green, because that color change might be achieved more simply by mutating the primary photoprotein. Perhaps GFPs evolved mainly to boost the overall quantum efficiency of emission. The chemiluminescence efficiencies of primary photoproteins are relatively low, but if their excited state energy can be efficiently transferred to a GFP that, in turn, emits with high efficiency, then the organism produces more light for a given energy cost (Refs 1, 8; W. W. Ward, M. W. Cutler and D. F. Davis, pers. commun.).

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Biochemical properties

Aequorea GFP is a protein of 238 amino acid residues. Its biggest absorbance peak is at 395 nm with a smaller peak at 475 nm. The amplitudes of these peaks (i.e. extinction coefficients) have been estimated as 21–30 and 7–15 $mM^{-1}\,cm^{-1}$, respectively (Ref. 8, W. Ward, pers. commun.). Excitation at 395 nm yields an emission maximum at 508 nm. The quantum yield, or probability of re-emitting a photon once the molecule has been excited, is 0.72–0.85 (Ref. 8; E. Kurlan, J. Rudzki-Small and F. Prendergast, pers. commun.), and the excited state lifetime is 3.25 ns (Ref. 10). To put these numbers in perspective, the well-known dye fluorescein has an extinction coefficient of 80 $mM^{-1}\,cm^{-1}$ at 490 nm and a quantum yield of 0.91. Because fluorescence brightness is proportional to the product of these numbers, wild-type *Aequorea* GFP excited with fluorescein filters is about an order of magnitude less bright than the same number of molecules of free fluorescein. Switching the excitation to 395 nm does not help because such wavelengths cause rapid photoisomerization (see below) and also excite more background autofluorescence.

The mature purified protein is highly stable, remaining fluorescent up to 65°C, pH11, 1% sodium dodecyl sulphate (SDS) or 6 M guanidinium chloride, and

resisting most proteases for many hours[11–13]. Formation of diffraction-quality crystals of GFP was reported in 1988[10], but an X-ray structure has not yet been published. The recent upsurge of interest in GFP is encouraging both crystallographic and nuclear magnetic resonance (NMR) investigations. An often overlooked feature of GFP is its tendency to dimerize, which is detectable as a partial suppression of the 475 nm excitation peak. This effect is promoted by high ionic strength and high protein concentrations and is roughly half maximal at 100 mM NaCl and 6 μM GFP[14]. The primary photoprotein aequorin binds to the dimer but not the monomer (W. W. Ward, M. W. Cutler and D. F. Davis, pers. commun.), and the dimer or even higher multimers would predominate in the light-emitting organelles of the marine jellyfish. Such direct binding would help the GFP grab the energy of the excited state of aequorin despite the modest amplitude of GFP's extinction coefficients.

Aequorea GFP shows no strong sequence homologies to other known proteins, though its chromophore and that of *Renilla* GFP are spectroscopically similar when denatured[15] and contain the same tripeptide sequence Ser-Tyr-Gly[5,16]. *Renilla* GFP is an even stabler protein than *Aequorea* GFP, is tightly dimerized unless denatured, and shows a single absorption peak at 498 nm (E = 270 $mM^{-1}cm^{-1}$ as dimer), with an emission peak at 509 nm and quantum efficiency of 0.8 (Ref. 15). The tremendous extinction coefficient of *Renilla* GFP indicates that it should be much brighter than *Aequorea* GFP; however, the yet stronger tendency to dimerize might complicate use of *Renilla* GFP as a fusion tag. Understanding and exploiting *Renilla* GFP awaits its cloning and high-level expression, which are now clearly worthwhile goals.

Maturation and fluorophore formation

Aequorea GFP owes its visible absorbance and fluorescence to a *p*-hydroxybenzylideneimidazolinone chromophore[5,17] formed by cyclization of Ser65, Tyr66 and Gly67 and 1,2-dehydrogenation of the tyrosine. The mechanism of this unique post-translational modification is not only of intrinsic biochemical interest but is also a crucial constraint on the speed with which GFP can report changes in gene expression. We think that cyclization probably comes first (Fig. 1) and would start by a nucleophilic attack of the amino group of Gly67 on the carbonyl group of Ser65 to form a five-membered ring, followed by loss of water to form an imidazolin-5-one intermediate (Fig. 1). The first step in this postulated reaction is closely analogous to the first step in the known deamination reaction of Asn-Gly sequences in peptides and proteins, in which the amino group of the Gly attacks the side-chain amide of Asn, also forming a five-membered ring[18]. If this analogy is correct, mutation of Gly67 in GFP should prevent or greatly hinder chromophore formation. Indeed, all known fluorescent mutants, as well as *Renilla* GFP, retain Gly at the appropriate position in the chromophore[16–19]. The three-dimensional structure of the protein probably provides further driving force for the cyclization by placing the amino group of Gly67 near the carbonyl group of Ser65.

We believe the final step in chromophore formation is the oxidation of the hydroxybenzyl side chain of Tyr66 by atmospheric oxygen. When GFP is expressed in anaerobically grown *Escherichia coli*, the protein is non-fluorescent. After oxygen is readmitted and fresh protein synthesis inhibited, fluorescence appears with a simple exponential approach to the final steady state, with a rate constant that is hardly affected by lysis of the bacteria and dilution of the cytoplasmic contents by ~10^5 (Ref. 20). The simplest explanation for the single exponential time course is that anaerobically expressed protein has already cyclized to the species labeled A in Fig. 1, and simply forms its chromophore once an oxidant is provided. In confirmation of this hypothesis, the molecular mass of

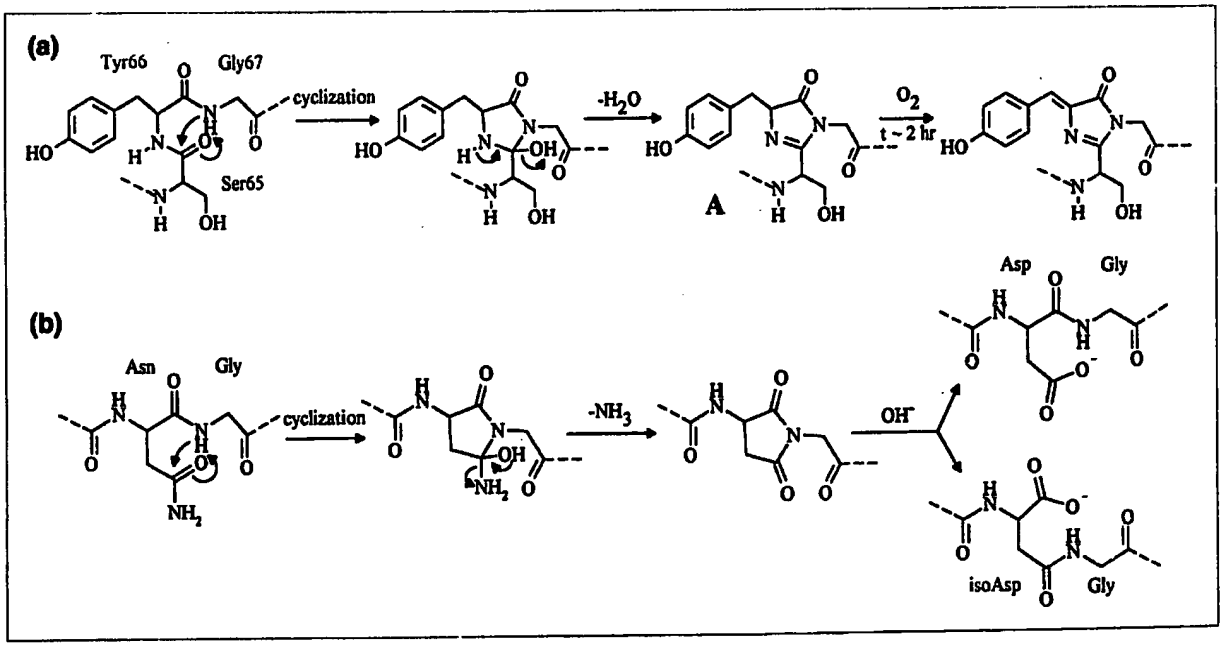

Figure 1

(a) Proposed biosynthetic mechanism for the green fluorescent protein (GFP) chromophore, in which cyclization precedes oxidation. Species **A** is what we believe to accumulate during anaerobic expression of GFP. **(b)** The accepted mechanism for hydrolysis of Asn-Gly sequences, whose initial cyclization step is quite analogous to that in the preferred mechanism for GFP.

anaerobically purified GFP, measured by electrospray ionization mass spectrometry, decreases by 1 ± 4 Da (mean ± standard error) after exposure to air and development of fluorescence (A. Boyd and L. Gross, unpublished). The proposed mechanism predicts a loss of 2 Da, whereas cyclization after oxidation would give a 20 Da loss. Measured time constants for the oxidative step in fluorophore development have been 2–4 h for wild-type GFP[10,20]. Inouye and Tsuji[21] have reported that dithionite reversibly reduces GFP to a nonfluorescent species; if this result can be reproduced, it would be interesting to see whether the reduced protein is species A. The mechanism of post-translational chromophore formation is of practical importance because it must remain functional in any useful GFP mutant or fusion, and because its kinetics may hinder attempts to measure rapid inductions of GFP as a reporter gene. We know almost nothing about the rate of cyclization because of the difficulty in monitoring its progress, though if the analogy to Asn-Gly deamidation is valid, its rate should increase with increasing pH[18]. Mutation can speed up the oxidation step (see below), but we do not know if enrichment of the oxygen concentration beyond that of ordinary air is effective.

Even after the chromophore is formed, it is fluorescent only when encapsulated inside the protein, where it is protected from the collisional quenching effects of oxygen[22] and water. Denatured protein or isolated peptides containing the chromophore absorb light but are practically nonfluorescent[15], presumably because the naked chromophore is neither rigid nor protected from jostling by solvent molecules. Also, the phenolic group on the chromophore is insensitive to external pH changes until the protein is beginning to unfold[11].

Photoisomerization and photobleaching

Photobleaching sets the ultimate limit on the amount of signal obtainable from any fluorescent marker. Irradiation of wild-type GFP can cause two distinct spectral changes over time: a photoisomerization that progressively decreases the 395 nm absorption peak and increases the 475 nm peak (Fig. 2), as well as a photobleaching process that causes both peaks to decline by approximately equal proportions. Photoisomerization dominates with excitation in the UV spectrum, especially near 280 nm, and may involve a UV-induced change in protein conformation that favors ionization of the phenolic remnant of Tyr66 in the chromophore. This effect is probably responsible for earlier observations by Chalfie *et al.* in that in *Caenorhabditis elegans*, exposure of GFP to 395 nm causes rapid loss of intensity at that excitation wavelength and results in greater fluorescence upon illumination at 475 nm[6]. In principle, photoisomerization could be usefully analogous to uncaging of fluorophores[23]. Diffusion or migration of GFP fusion proteins can be monitored after spatially localized photoisomerization[24], although one suspects that the contrast could bear improvement.

After photoisomerization is complete, or upon illumination at 475 nm, only slow photobleaching is observed. The rate of photobleaching of wild-type GFP has been reported to be about half that of fluorescein (W. W. Ward, M. W. Cutler and D. F. Davis, pers. commun.). Because the extinction coefficient is much less than half that of fluorescein, the photobleaching quantum yield is presumably considerably higher than that for fluorescein.

Mutating GFP to alter and improve its properties

Most mutations in GFP result in a partial or complete loss of fluorescence without significant change in relative absorption or emission peaks. These mutations probably cause misfolding of the protein, failure of chromophore formation, or quenching of the fluorescence by insufficient shielding. Attempts to truncate the gene have shown that only one residue can be sacrificed from the amino terminus and less than 10 or 15 from the carboxyl terminus before fluorescence is lost (Ref. 25; W. W. Ward, M. W. Cutler and D. F. Davis, pers. commun.). The intolerance

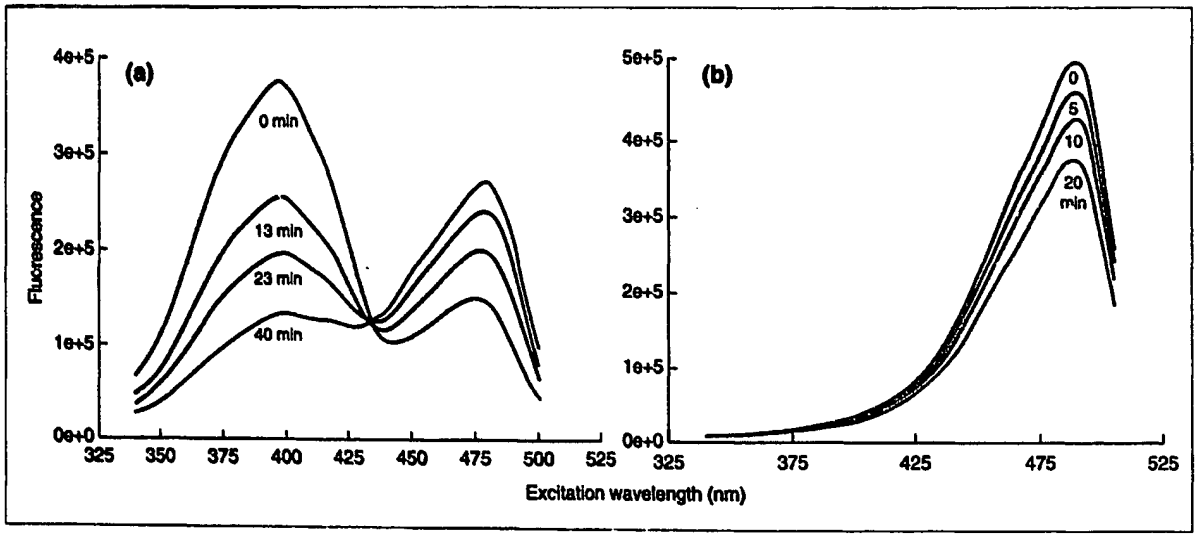

Figure 2

Behavior of wild-type green fluorescent protein (GFP) **(a)** and the S65T mutant **(b)** upon progressive irradiation with ultraviolet light. GFP samples were illuminated at 280 nm from a xenon lamp and monochromator, with constant intensity for both samples. At the times indicated, excitation spectra were recorded at the emission peak wavelengths (508 nm for wild-type and 510 nm for S65T). Wild-type GFP suffered photoisomerization, decreasing the excitation amplitude at 395 nm while increasing the amplitude at 475 nm. This effect was not reversible upon placing the GFP in the dark. The S65T mutant showed no photoisomerization, only slow bleaching.

of GFP to major truncation is perhaps not too surprising, because the protein scaffold must both synthesize the chromophore and rigidly shield it from the surrounding water.

A subset of mutations affect the relative ratio of absorption peaks, at 395 and 475 nm, presumably promoting or hindering deprotonation of the chromophore. Currently, the clearest examples are T203I (Thr203→Ile) and E222G (Glu222→Gly), which simplify the spectra to single absorption peaks at either 395 or 475 nm, respectively[26]. The mutation I167T (Ile167→Thr) inverts the wild-type ratio of the two peaks without eliminating either completely[20].

A second subset of mutations produce essentially new excitation and emission spectra with significantly altered characteristics. So far, the main examples of this type of mutation are within the chromophore region itself. Replacement of the central tyrosine (Y66) by other aromatic amino acids (Trp, His or Phe) shift the excitation and emission spectra to progressively shorter wavelengths (see Table I). These point mutants are distinctly less fluorescent than wild-type GFP, presumably because the alternative amino acids fit less well into the central cavity. The fluorescence spectra of the histidine mutant Y66H are not sensitive to pH changes until the protein is on the verge of denaturation, which provides additional evidence that the chromophore is inaccessible to solvent.

Replacements of Ser65 by Thr, Cys, Leu, Val and Ala result in spectra that have lost the 395 nm absorption peak and in which the remaining 475 nm peak is shifted to slightly longer wavelengths[9]. These spectra are now quite similar in wavelength and shape to that of *Renilla* GFP[16]. The longest-wavelength peaks (490 nm excitation, 510 nm emission) arise from S65T, which has three other striking advantages: (1) about sixfold greater brightness than wild-type when each is excited at its longest-wavelength peak; (3) fourfold faster oxidation to the final fluorescent species than wild-type[9]; (3) no photoisomerization and only very slow photobleaching (Fig. 2b). Our preliminary findings indicate that S65T photobleaches at about 1/7 the rate of fluorescein at 488 nm irradiation in air-saturated buffer at pH7.1. Because the extinction coefficient of S65T is about 4/7 that of fluorescein under these conditions, the quantum efficiency of photobleaching of S65T may be calculated to be about 1/4 that of fluorescein. These advantages make S65T more attractive than wild-type GFP for most applications except those in which long-wave UV excitation or photoisomerization is essential. Of course, further mutations may yet give additional improvements. GFP may be a good test bed for directed protein evolution and exploration of how large numbers of substitutions interact to influence protein properties[27], because it is a single polypeptide chain easily expressed in bacteria, and differences in its wavelengths or energy levels are easily and accurately quantifiable *in situ* without isolating the protein.

Table I. The effect of mutations within the chromophore region of GFP

Mutation	Excitation maximum (nm)	Emission maximum (nm)
Mutations at Ser65		
Wild-type	396, 475	508[a]
S65A	471	504
S65C	479	507
S65L	484	510
S65T	488	511
Mutations at Tyr66		
Wild-type	396, 475	508[a]
Y66F	360	442
Y66H	382	448
Y66W	436	485

[a]Emission maximum value when excited at 396 nm. Emission maximum value is 503 nm when excited at 475 nm.

Youvan and colleagues[19] have performed extensive random mutagenesis of residues 64–69 and isolated six mutants whose spectra are qualitatively similar to the S65 mutants described above. Four of them have the same substitutions (Leu, Cys or Ala) at position 65 as listed above. If any of the mutants are verified to offer similarly increased brightness, faster fluorophore formation and simpler photochemistry than wild-type, they should be excellent alternatives for use as a research tool.

Applications

Table II lists some of the presently known applications for GFP. This list must be incomplete given that many of the newest and most innovative applications of GFP are not yet published. Many short reviews heralding the usefulness of GFP have already appeared[3,24,28–31]. A fluorescent protein newsgroup has been formed on Bionet (see Box at the end of this article), which may prove the fastest means for disseminating information about GFP.

GFP as a marker of gene expression and cell lineage

As far as we know, GFP has been successfully expressed at least once in every species that it has been tried in, although there are also many anecdotes or rumors of failures in specific contexts. Polyclonal antibodies against GFP that work in western blots and immunocytochemistry are easy to raise and are quite helpful in troubleshooting because they can detect GFP even if it fails to become fluorescent or has been denatured (R. Heim, R. Tsien and C. Zuker, unpublished). Antibodies are also commercially available (e.g. from Clontech). Most of the successful visualizations of wild-type GFP in mammalian cells seem to have required strong promoters such as that from cytomegalovirus[32,33]. Expression of GFP in organisms that grow at cooler temperatures has been relatively less problematic, with good expression in plants[34], yeast[24] and *Dictyostelium*[35], and some successes in *Drosophila*[36–38]. In yeast and HeLa cells, GFP expressed at 37°C is many times less fluorescent than that expressed at 15°C. Heat acts mainly by causing improper maturation rather than by decreasing expression levels or the brightness of properly matured GFP[39]. This effect has been ingeniously exploited to track the fate of GFP formed before and after a temperature shift[39,40]. In mammalian cells, cooling to 33°C is enough to improve the GFP signal noticeably[33]. In *Arabidopsis* but not in maize, expression requires codon swapping to remove an undesirable splice site[34,41].

Although many of the weaknesses of wild-type GFP can be ameliorated by use of mutants such as S65T, GFP fundamentally lacks one stage of amplification built into a true enzymatic

Table II. Examples of applications of GFP

Organism	GFP version[a]	Fusion protein	Linker of GFP	Terminus[b]	Comments	Reference
Saccharomyces cerevisiae	WT	Histone H2B	None	N	Localizes to nucleus	J. Loeb, unpublished
S. cerevisiae	WT	SRP1 nuclear import receptor	None	C	Complements null localized to nuclear envelope	J. Loeb, unpublished
Schizosaccharomyces pombe	S65T	Calmodulin	$(GA)_5$	C	Functional and localized OK	T. Davis, M. Moser and R. Stevens, unpublished
S. cerevisiae	S65T	Calmodulin	$(GA)_5$	C	Functional and localized OK	T. Davis, M. Moser and R. Stevens, unpublished
S. cerevisiae	S65T	MYO2 class V unconventional myosin	$(GA)_5$	C	Functional and localized OK	T. Davis, M. Moser and R. Stevens, unpublished
S. cerevisiae	S65T	NUF1 component of spindle pole body	$(GA)_5$	C	Cells grow more slowly	T. Davis, M. Moser and R. Stevens, unpublished
S. cerevisiae	WT S65T	Actin	0.4 or 10 amino acids (Ala)	N & C	Localization improves with spacer length. Carboxy-terminal fusions do not complement null mutations but are localized to actin cortical patches	T. Doyle, unpublished
S. cerevisiae	S65T	STE2 receptor	Glu–Phe	N	Functional	S. Ishida and H. Bourne, unpublished
S. cerevisiae	WT	TUB1 α-tubulin TUB4 γ-tubulin	None	N & C C	Correctly localized but do not rescue null mutations	T. Stearns, unpublished
S. cerevisiae	WT	FUS1	None	N	Localizes to mating projection	S. Kron, unpublished
S. cerevisiae	WT	JNM1 (dynein motor)	None	N	Functional	J. McMillan and K. Tatchell, unpublished
Drosophila	WT	None	None	–	Expression under control of GAL4 enhancer trap	Yeh, E., Gustafson, K. and Boulianne, G. L. (1995) *Proc. Natl Acad. Sci. USA* 92, 7036–7040
Drosophila	WT	None	None	–	Marker of indirect flight muscle development	Barthmaier, P. and Fyrberg, E. (1995) *Dev. Biol.* 169, 770–774
Drosophila	WT S65T	None	None	–	Expression under control of glass gene promoter	Plautz, J. D. *et al. Gene* (in press)
Xenopus	WT	None	None	–	Lineage marker	Tannahill, D., Bray, S. and Harris, W. A. (1995) *Dev. Biol.* 168, 694–697
Potato plant	WT	None	None	–	Expression through infection with potato virus–GFP construct	Baulcombe, D. C., Chapman, S. and Cruz, S. S. (1995) *Plant J.* 7, 1045–1053
Soy bean	WT S65T	None	None	–	Removal of cryptic splice site improves expression	Plautz, J. D. *et al. Gene* (in press)
Nicotiana benthamiana	WT	Movement protein of tobacco mosaic	None	N	Desired functions and targeting normal	H. Padgett, M. Heinlein, B. Epel and R. Beachy, unpublished
Maize protoplasts	WT	None	None	–	Cauliflower mosaic virus 35S promoter	Hu, W. and Cheng, C. L. (1995) *FEBS Lett.* 369, 331–334

reporter system in which each protein molecule can generate thousands of chromophore or fluorophore molecules. Because each GFP represents one fluorophore, relatively high levels of GFP expression, as much as 10^6 molecules per cell[32], may be necessary to give bright signals. Furthermore, properly matured GFP is a stable protein and might not readily reflect dynamic bidirectional changes in gene expression. These problems may be less significant for using GFP as a long-term lineage marker, provided GFP can be expressed from a strong, cell-type-specific promoter. The use of different colored GFPs to track multiple cell types enables differences in

Table II. contd

Anabaena (Cyanobacteria) (PCC7120)	WT	None	None	–	Marked toxicity when highly expressed	W. Buikema, unpublished
Caenorhabditis elegans	WT S65T S65C	Myosins GLP-1, HLH-1 SKN-1, CEY-2	None	N	WT GFP fluorescence in differentiated tissues only S65T and S65C fluorescence in undifferentiated embryos	A. Fire, J. Ahnn, G. Seydoux and S. Q. Xu, unpublished
C. elegans	S65T S65C	SV40 nuclear localization signal	None	N	Sorts to nucleus correctly	A. Fire, J. Ahnn, G. Seydoux and S. Q. Xu, unpublished
C. elegans	S65T S65C	Synthetic secretion signal	None	N	S65T may be more fluorescent when secreted S65C may be more fluorescent intracellularly	A. Fire, J. Ahnn, G. Seydoux and S. Q. Xu, unpublished
C. elegans	S65T S65C	Mitochondrial matrix localization signal	None	N	Results in marked enhancement of fluorescence in early embryos	A. Fire, J. Ahnn, G. Seydoux and S. Q. Xu, unpublished
C. elegans	S65T S65C	LacZ	None	C	Less fluorescent, poor solubility	A. Fire, J. Ahnn, G. Seydoux and S. Q. Xu, unpublished
C. elegans	WT	pat-3 integrin	N	3 amino acid residues	Rescues null	
COS cells	S65T	Protein tyrosine phosphatase μ	None	N	Desired functions and targeting normal	G. Zondag, M. Gebbink, and W. Moolenaar, unpublished
HeLa cells Pig LLCPK	WT S65T	Inner centromere proteins	6 Gly	N	Functional	A. Ainsztein and B. Earnshaw, unpublished
CHO cells	WT S65T	None	None	–	S65T fluorescence 100 times greater than WT	J. Tavare, unpublished
HeLa cells	WT	CENP-B centromere protein	None	–	Functional	K. F. Sullivan, K. M. Hahn, and R. D. Shelby, unpublished
HeLa cells	WT	Mitochondrial targeting sequence	Yes	N	Correctly targeted to mitochondria	Rizzuto, R. *et al.* (1995) *Curr. Biol.* 5, 635–642
HeLa cells	S65T	Chromogranin B	Val-Pro	N	Moves from ER to Golgi then secreted into medium, dependent on temperature	Kaether, C. and Gerdes, H. H. (1995) *FEBS Lett.* 369, 267–271
GH3 cells	WT S65T	None	None	–	High level expression toxic	Plautz, J. D. *et al. Gene* (in press)
BHK cells	WT	MAP4	None	–		Olson, K. R., McIntosh, J. R. and Olmsted, J. B. (1995) *J. Cell. Biol.* 130, 639–650
HEK 293 (Human embryonic kidney cells)	Y66H WT I167T	Glutamate receptor subunits GluR3 and GluR6 NMDAR1	None	C	Glutamate-gated ion channel appears normal and localized to surface. Y66H fluorescence not detectable	Marshall, J. *et al.* (1995) *Neuron* 14, 211–215
Transgenic mice	WT	None	None	–	CMV-IE enhancer, actin promoter; non-toxic	Ikawa, M. *et al.* (1995) *Dev. Growth Differ.* 37, 455

[a]WT, wild-type.
[b]N, amino terminus; C, carboxyl terminus.

cell movement or migration to be visualized in real time without the need to add additional agents or fix or kill the cells. Figure 3 presents such an example of tracking of the several cell types in the slime mold *Dictyostelium discoideum*. This is particularly valuable in developmental biology where spatial cues and differential sensitivities to chemoattractants can lead to altered patterns of cell movement and fate.

GFP as a protein tag

The ideal fusion of GFP with a host protein preserves both the fluorescence of GFP and all the targeting and physiological functions of the host protein. This is an application, unlike

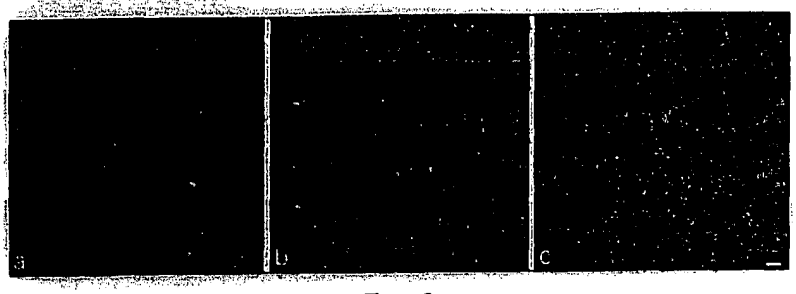

Figure 3

A top-down view of a chimeric mound of *Dictyostelium* cells: one population is marked with wild-type green fluorescent protein (GFP), another population with the blue-emitting Y66H mutant. Each panel is a projection along the optical axis of two three-dimensional images (a green and a blue), each consisting of 64 focal planes collected with a cooled charge-coupled-device (CCD) camera and processed to remove out-of-focus light (see Ref. 43 for details). Panels **a**, **b** and **c** indicate cell positions at 10 min intervals. Bar = 10 μm. Data courtesy of I. Reddy and J. G. McNally, University of Washington, WA, USA.

detection of gene expression, in which GFP is truly unique. Both the amino and carboxyl terminii have been successfully fused to a wide range of cytosolic and membrane-bound proteins. Fusions in which the amino terminus of GFP has been fused at the carboxyl terminus of the host protein have generally worked without flexible linkers. The likelihood of successful fusion of proteins to the carboxyl terminus of GFP might be enhanced by linker sequences. A range of GFP–host fusion proteins is presented in Table II.

Monitoring protein–protein interactions

Fluorescence resonance energy transfer (FRET) is a general, non-destructive, spectroscopic method that can monitor the dynamic association of macromolecular partners in living cells[42]. FRET is predictable, quantitative, can be imaged in single cells with high temporal and spatial resolution and, unlike the two-hybrid system, does not require transplantation into yeast nuclei. However, it needs a general method for labeling one macromolecular participant with a donor fluorophore and the other with an acceptor fluorophore, such as expressing fusions of the two prospective partners with different-colored GFP mutants. The emission spectrum of the donor must overlap significantly with the absorption spectrum of the acceptor, while the overlaps between the two absorption spectra and between the two emission spectra should each be minimized. For example, the blue mutation Y66H can be the donor (excitation maximum 382 nm, emission max. 448 nm), while mutants S65C or S65T can be the acceptor (excitation max. 479 or 489 nm, emission max. 507 or 511 nm) (Fig. 4). Wild-type GFP would not be a favorable acceptor, because UV excitation of Y66H near its 382 nm absorption peak would also directly excite wild-type GFP at its 395 nm absorption peak without any energy transfer. We have verified that FRET does occur between Y66H and S65C when fused together via a cleavable 25-residue spacer (R. Heim, unpublished). Ultimately, one would prefer to avoid UV excitation and use something like S65T as a donor and a green-absorbing, yellow- or red-emitting mutant as the acceptor, but the latter has yet to be devised.

Concluding remarks

GFP is a tool of enormous potential but, like most such powerful technologies, its mechanism of operation and limitations need to be evaluated critically. Although the majority of investigators currently trying GFP are mainly interested in monitoring gene expression or protein localization, considerable re-engineering of GFP based on an understanding of the protein may prove valuable or necessary for more sophisticated applications.

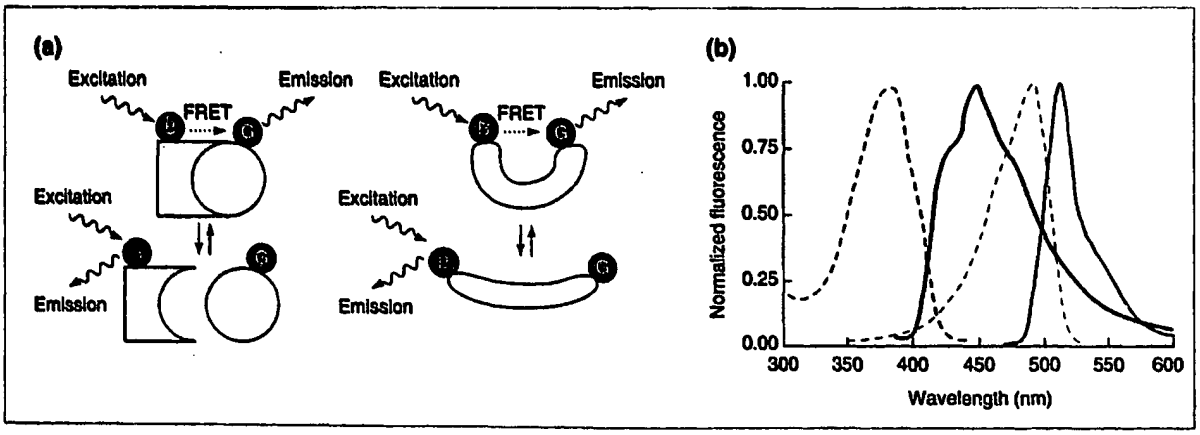

Figure 4

Possibility of monitoring protein–protein proximity by fluorescence resonance energy transfer (FRET) between differently colored mutants of green fluorescent protein (GFP). **(a)** Energy transfer from a blue-emitting mutant (Y66H) to an improved green mutant (S65T) of GFP depends on the distance between the fluorophores and thereby might monitor the proximity of the host protein subunits or domains. **(b)** Spectral overlap between GFP mutants Y66H and S65T. Fluorescence spectra of Y66H (blue lines) and S65T (green lines) show good overlap between the Y66H emission (solid blue line) and S65T excitation (dotted green line) spectra, a prerequisite for FRET. We currently estimate 40 Å as the characteristic distance R_0 at which energy transfer between randomly oriented fluorophores would be 50% efficient. All spectra were normalized to unit peak amplitude for ease of comparison. Before normalization, the peak absolute intensity from S65T was about 18 times that from Y66H for equal amounts of purified protein, a disparity that still needs to be reduced by further mutagenesis.

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Fluorescent Protein newsgroup on Bionet

Interested participants in Europe, Africa, or Central Asia may subscribe by sending the command 'SUB bionet-news.bionet.molbio.proteins.fluorescent' to MXT@dl.ac.uk. Participants in the Americas or the Pacific Rim should send the message 'subscribe fluorpro' to biosci-server@net.bio.net.

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